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The mechanism of formation of lipid tubules from liposomes

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Certain diacetylenic phospholipids form liposomes in water above their chain melting transitions, which, if slowly cooled, quantitatively convert to hollow tubular structures about 1 μm in diameter and as long as hundreds of micrometers. To elucidate the nature of the conversion process, freeze fracture electron microscopy was utilized to examine samples that were rapidly quenched during tubule formation. Many transitional structures were observed, typically liposomes partially wrapped around nascent tubules. This is consistent with real-time imaging by optical microscopy indicating tubule growth by continuous transfer of lipid bilayers from liposomes by a rolling-up process. The mechanism of the conversion process, combined with preliminary X-ray scattering data indicating unusual packing of the lipid molecules, suggests an explanation for the efficiency of the conversion process and why the tubule is a favorable microstructure for the crystalline lipid.

Keywords: tubule; polymerizable lipids; liposomes; microscopy; formation

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Introduction

We have been studying the properties of polymerizable lipids, in particular one of a class of lipids that form unusual tubular structures [1–6]. The lecithin 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{23}PC$) contains a diacetylenic group in the middle of each hydrocarbon chain that renders it and similar lipids polymerizable by ultraviolet light and other forms of radiation [7–14]. $DC_{23}PC$ forms liposomes in aqueous dispersion above its phase transition temperature of 43 C that are unstable at lower temperatures [1, Burke et al., unpublished]. If the temperature is lowered to about 38 C, most liposomes convert to hollow the "tubules", whose structure has been described at some length [2]. These are 0.3–1 μm in diameter, tens or even hundreds of micrometers in length, and have walls that vary from two to about ten bilayers in thickness. Tubule ends are generally open, unless they contain trapped liposomes (Fig. 1). When liposomes are trapped they allow immobilization of aqueous contents within the tubule [15]. Tubules without trapped liposomes can be produced by precipitation from ethanolic solution – a method that can also generate some precursor helical structures but provides good control of tubule dimensions [6]. The diamagnetism of the hydrocarbon chains also allows tubules to be oriented by strong magnetic fields [16].

Polymerization occurs in diacetylenic systems only when the reactive groups are in a crystalline array with a particular range of spacing and orientation [17]. Since ultraviolet irradiation of the tubules produces colored polydiacetylenic chains [1], the lipid hydrocarbon chains in the tubule phase must have high local order and appropriate

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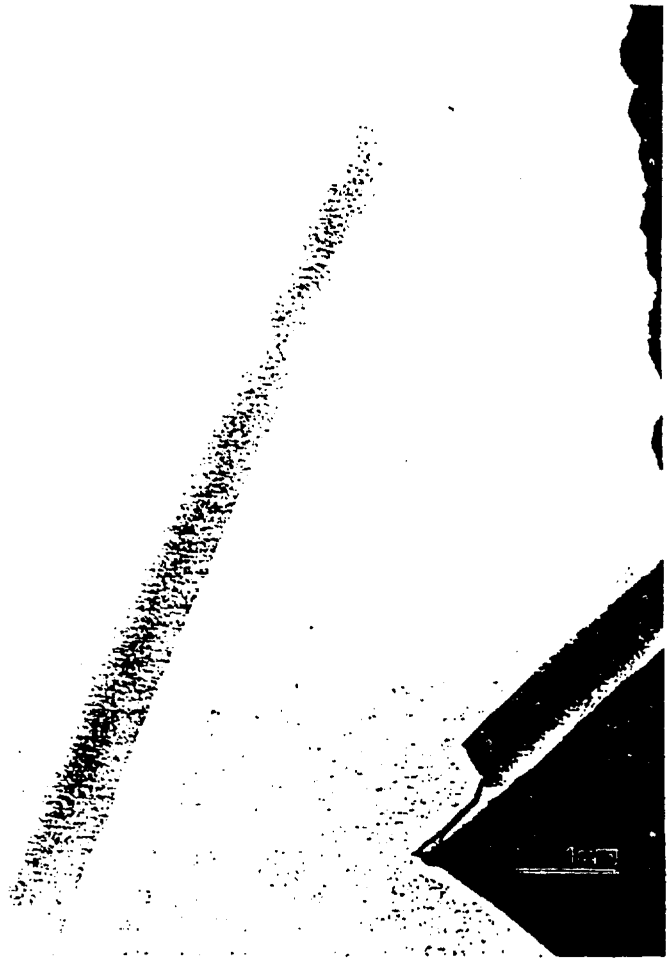

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Fig. 1. Freeze fracture electron micrograph of a typical $DC_{23}PC$ tubule made from liposomes in distilled water. The tubule in this micrograph has a diameter of 0.8 μm. and a length of 15.4 μm. Note the wrapping of double bilayers around its circumference, and that one end appears to be occluded by at least one bilayer. The dark area to the side of the tubule is lipid material not removed by cleaning. Scale bar, 1 μm.

relative chain positions. The straightness of tubules probably also reflects ordered molecular packing. Based on data from Raman and infrared spectroscopy [13,14,18 Burke et al., unpublished], recent polarized light microscopy and preliminary electron diffraction experiments, we will refer to such order as "crystallinity". By comparison, the monomeric liposomes that exist above 43°C have disordered hydrocarbon chains [14].

That tubule walls are constituted of bilayers has been supported by the stability of the $DC_{23}PC$ tubule structure in water and the fact that when tubules frozen in ice are fractured they cleave along what appear to be bilayer midplanes. However, there has been no direct evidence that bilayer structure is maintained during the drastic morphological change during conversion of liposomes to tubules.

Optical microscopic observation of the formation and growth of tubules suggests two mechanisms: crystallization of monomers lost by cooling liposomes or wrapping pre-existing liposomal bilayers. The rapid lengthening of tubules during growth strongly resembles the precipitation of crystals from solution. However, the extremely low critical micelle concentration for lecithins with long hydrocarbon chains in water seems to rule out a mechanism in which the tubules grow at the observed rate by addition of monomers from a purely aqueous solution. During growth tubules often appear to pull material from large liposomes with which they are in contact, resulting in transient distortions of the shapes of the liposomes. Tubules may occasionally be seen to wobble as they grow, perhaps reflecting their rotation as they acquire material from the liposomes. The resolution of optical microscopy is not sufficient to prove continuity of liposomal bilayers with those on growing tubules. Electron micrographs of replicas of samples quenched after equilibration at 20 C (see Fig. 2) show strong evidence that tubules grow by wrapping themselves in accreting sheets of paired bilayers. To determine if wrapping of liposomes occurs during tubule formation we trapped intermediate states in the conversion process by rapid freezing of converting samples, followed by freeze fracture electron microscopy.

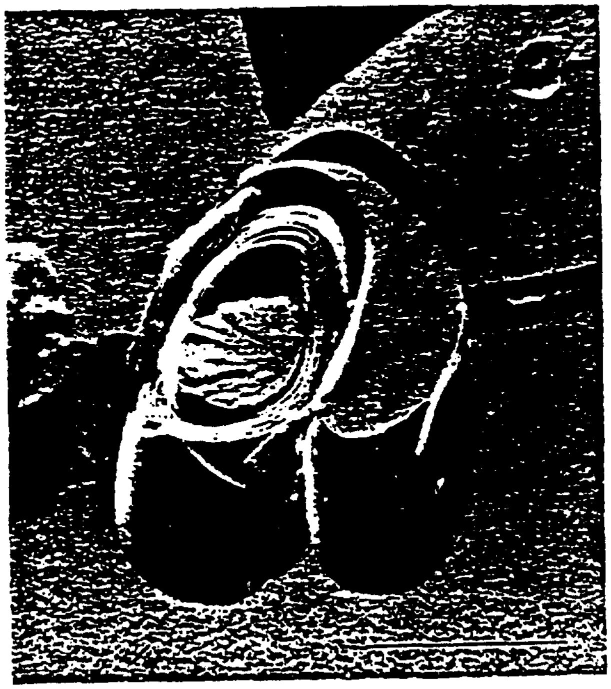

Fig. 2. Freeze fracture electron micrograph of a liposome wrapped around a tubule, reminiscent of a glial cell laying myelin around a nerve axon. This sample was prepared identically to that in Fig. 1. This and subsequent micrographs have been printed with "dark shadows" - with an internegative between the plate from the microscope and the final print. Scale bar, 0.5 μm.

Methods

DC$_{23}$PC was synthesized (using egg lecithin as the source for the headgroup) and purified in our laboratory by previously published methods [7]; it gave a single spot by thin-layer chromatography on silica gel in a chloroform/methanol/water system. It was dried from chloroform by a dry nitrogen stream, and residual solvent was removed under vacuum for 12 h. Following hydration with distilled water in an incubator at 60 C, samples were stored frozen at −20 C. A simple device was assembled to allow the rapid freezing of growing tubules consisting of a copper plate soldered to tubing through which water was circulated. Temperatures at various locations on the plate were monitored with a small thermocouple. Immediately before use hydrated lipid was warmed in the 55°C water bath for several minutes to allow full reformation of liposomes. A small aliquot of this dispersion was transferred to a Balzers (Hudson, NH) specimen holder on the 45°C portion of the warming plate. The specimen holder was then moved to a portion of the plate at 38°C that could be observed by a stereomicroscope. Within a few minutes the initiation of tubule formation could be seen as increased turbidity in the sample droplet. At the first such sign the holder was plunged into melting nitrogen and subsequently stored at −196°C until ready for fracturing.

Glycerol (10%) was added to other samples immediately prior to freezing from room temperature. All samples were transferred to a Balzers BAF 400D freeze fracture unit, fractured, and then replicated at −110°C and 2×10^{-6} Torr. Replicas, which were made with 2.5 nm Pt-C and 15 nm of C, were cleaned on ethanol and chloroform, mounted on 200 mesh grids, and photographed on Philips EM200 (Philips Electronic Instruments, Inc., Mahwah, NJ) or Zeiss EM-10 (Carl Zeiss Inc., Thornwood, NY) transmission electron microscopes.

Results and discussion

The replicas of quenched samples (Figs. 3 and 4) show complete well-ordered tubules and liposomes as well as what we infer to be intermediate states

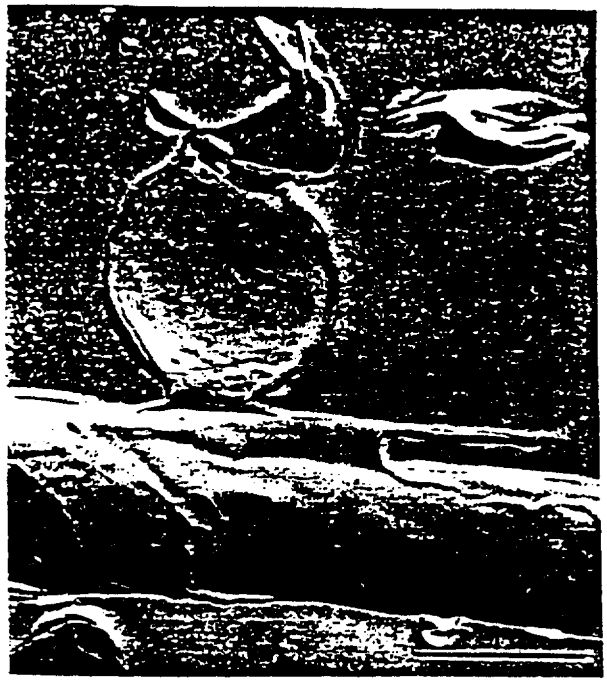

Fig. 3. Freeze fracture electron micrograph of a sample quenched during the conversion process as described in the text. Note the central liposome whose constituent bilayers are wrapping around two different nascent tubules. Scale bar, 0.5 μm.

between liposomes and tubules. These include tubules with liposomes wrapping around them, disordered tubules that appear to have irregular liposomal sections attached to them, and growing tubules linked to adjacent liposomes by continuous pieces of bilayer. These intermediate structures would not occur if tubules grew by accretion of monomers from solution. In all observed cases bilayers in the form of flattened cylinders wrap around tubules; consequently bilayers add to tubules in pairs. Odd numbers of bilayers in tubule walls (as observed, for example in Ref. 2, Fig. 5) probably result from trapping of liposomes within growing tubules, which can clearly be seen in Fig. 1. The mechanism by which water leaves the converting liposomes is not revealed by these micrographs, and is best studied by other methods.

We presume that the driving force for tubule formation is related to rearrangement within the bilayer during formation of regions of crystalline hydrocarbon chains. There is no evidence from electron microscopy indicating when the bilayer phase transition takes place relative to the addition of material of the tubule, but rigid "crystalline"

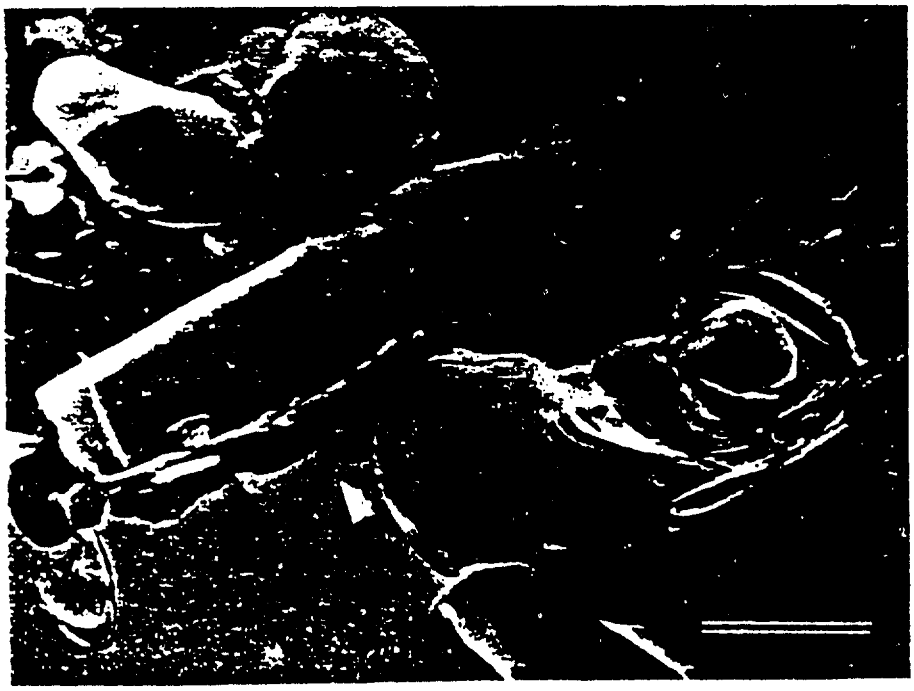

Fig. 4. Freeze fracture electron micrograph of several adjacent structures present in a quenched sample. Note the presence of liposomes, well-formed tubules, and several intermediate structures, all contiguous and apparently sharing the bilayer material. Scale bar, 0.5 μm.

bilayers probably could not easily deform from the liposomal to the tubular geometry as observed during wrapping. The formation of tubules, associated as it is with a crystallization, is exothermic; the violent motion often seen in the optical microscope during tubule formation suggests that some of the released energy is converted to translation and rotation energy of the forming tubules.

In systems such as cochleate cylinders, attraction between successive bilayer planes has a strong role in producing the solid microstructure [19]. Tubules, on the other hand, are hollow and we have often seen appreciable aqueous spaces between successive wraps of the wall material, particularly when they are formed in glycerol/water mixtures (see Fig. 5), implying that the driving force is generated within single bilayers.

We have not identified any structures from micrographs that definitely represent nucleation sites for the formation of tubules. In optical microscopic observations we have never seen tubules begin growing from isolated liposomes, but frequently from contacting areas between liposomes and between liposomes and tubules. The nucleation events, which probably involve contact between liposomes, must be short-lived, and are consequently unlikely to be observed by freeze fracture techniques.

The freeze fracture data describe but do not explain the phenomenon of tubule formation from cooled $DC_{23}PC$ liposomes. The recent publication by Helfrich [20] of a theory of helical wrapping of bilayer strips composed of chiral molecules is helpful, but inadequate. While we do often see regular narrow strips of lipid in the walls of tubules [2], the theory does not explain why a cylindrical structure would be formed from bilayer sheets whose widths were not constant. While wound strips of lipid bilayer in the tubular geometry appear to be stable, so too are the smooth continuous cylinders with the same dimensions that have no edges that are also observed. The following are speculations toward an explanation of tubule stability, nucleation, and subsequent growth either by elongation of addition of layers to pre-existing tubular sections.

The regular cylindrical structure of the tubule wall is strongly suggestive of crystal packing that supports a finite curvature in one direction and no curvature in another. Packing constraints on even achiral molecules in a single crystalline monolayer could produce a bias toward positive curvature in one direction only, but apposition of two such monolayers would appear to nullify the tendency. However, any pre-existing curvature in a bilayer breaks the symmetry about the bilayer midplane, so as long as this initial asymmetry promotes slightly different packing on the inner and outer monolayers, the cylindrical bilayer can be stable. Also, if the intermolecular packing in one or both monolayers were similar to that in cholesteric liquid crystals, this could generate an inherent helical twist to bilayers with a specific handedness. The formation of long regular cylinders by helical association of chiral biopolymers such as proteins is very well established – microtubules and the tobacco mosaic virus are but two examples. It should not be surprising that lipid bilayers could favor a cylindrical geometry based on a helical packing of lipid molecules.

When dispersions of phosphatidylcholines with two saturated hydrocarbon chains crystallize, there is a decrease of as much as 20% in the surface area of the liposome [21,22]. The contraction is roughly isotropic and causes neither topological nor major geometrical changes, although for large multilamellar liposomes the mechanical deformations take several seconds, limiting the rate of crystallization of the hydrocarbon chains [23]. The more drastic conversion of diacetylenic lecithin liposomes to tubules can take several minutes to go to completion. Unusual packing of the diacetylenic hydrocarbon chains in the crystalline phase might be the cause of the slow rate of conversion, the unusual tubular product of that conversion, and hysteresis in the phase transition [15].

The electron density profile along the axis normal to the $DC_{23}PC$ tubule bilayer has recently been obtained from low angle X-ray scattering (Rhodes et al., unpublished). Principal maxima correspond to phosphate, headgroup, and acetylene electron-dense regions. The phosphate-to-phosphate distance in tubules at 15°C was found to be only 49.5 Å, comparable to the value of 48.8 Å determined for dipalmitoyl phosphatidylcholine, which is 7 methylene units shorter [24]. In order for the $DC_{23}PC$ chains to be accommodated, the chain

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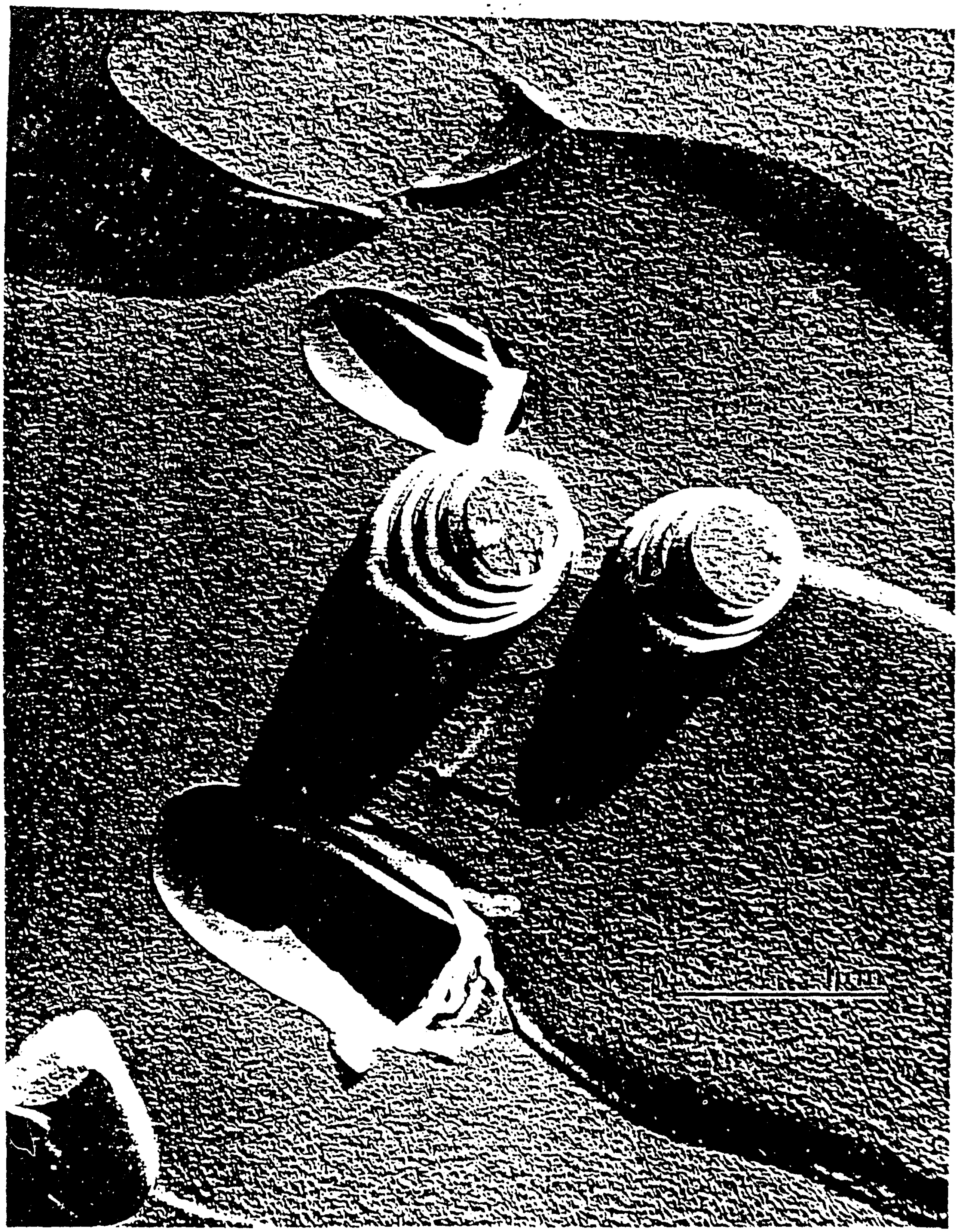

packing must allow partial interdigitation of the chains, tilting of the molecules, or some combination of the two. Without interdigitation, the molecules would have to tilt at approximately 45° relative to the bilayer normal – an angle much steeper than that in other crystalline phospholipid structures. This observation, and the fact that the acetylene-acetylene spacing (~15 Å) is so small, strongly suggest that the chains are tilted and partially interdigitated. Full data on monomeric and polymerized tubules will be presented elsewhere (Rhodes et al., unpublished).

This anomalous crystal packing suggests a mechanism for formation of unusual structures. If all chains in the $DC_{23}PC$ crystal were to tilt in the same direction, there could be an increase in the headgroup spacing in the direction of tilt as the hydrocarbon chains crystallize on cooling, resulting in lengthening of the bilayer (see Fig. 6) along the tilt axis. If in the crystallized monolayer there were a direction in which the molecular spacing were larger than in the melt, then a supercooled fluid monolayer, when placed under anisotropic tension, would crystallize by elongating along the lines of the tension. This is a manifestation of the well-known phenomenon of strain-induced crystallization in polymers. Crystallization perpendicular to the tilt direction would be disfavored by tension, so if the system were to remain under tension, all of the material in the original monolayer could convert to a narrow strip of crystalline lipid. Narrow crystalline strips in certain mixed phospholipid monolayers apparently can be stable even at equilibrium (Singh et al., unpublished).

Given this hypothesis, nucleation of tubule formation from a slightly supercooled liposome would occur in the presence of anisotropic lateral tension or compression in the plane of the monolayer. A liposome can deform to avoid compression, but tension can be generated by sticky collisions between two objects, be they two liposomes or a liposome and an existing tubule. Once in contact, relative motion generates tension at the contacting surface, nucleating a lengthening strip of crystalline bilayer from one liposome (see Fig. 7). In the case of collision between two liposomes, it is possible that one of them may be trapped in the tubule interior.

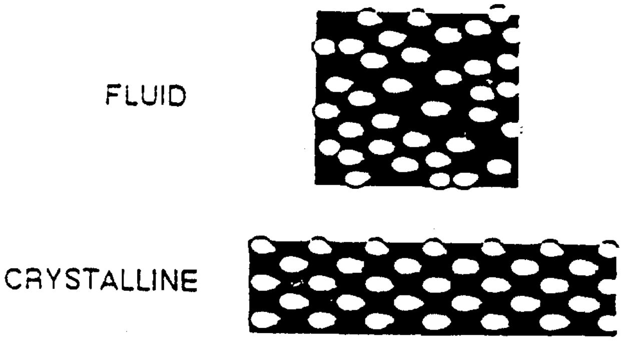

Fig. 6. A comparison of proposed headgroup packing by lipid molecules in fluid and crytalline states if the head group spacing is large in one crystal axis. The number of molecules and surface areas in both phases are shown as roughly equal.

Several explanations have been suggested for why crystallizing $DC_{23}PC$ should roll onto a tube. The lipid crystal structure may favor a helicial geometry of a particular pitch that would lead to generation of cylinders of a fixed diameter. Alternatively, Helfrich has predicted [20] that a strip of bilayer could form a regular helix solely because of tensions created by interaction of chiral molecules with bilayer edges. Tubules made of wound strips of bilayer about 1 μm in width are, in fact, often seen (see, for example, Ref. 2, Fig. 2). We believe such strips to be flattened tubes, but their existence is consistent with Helfrich's theory.

An alternative approach is to find the lowest energy route for the extension of a sheet of bilayer next to a large object with great hydrodynamic drag like a liposome. The new surface area should be taken up in a way that minimizes relative translational motion of large objects. Rolling of the new material into a cylinder satisfies this criterion. A tubule would undergo rotational diffusion along its long axis, with one rotational direction favored because any addition of crystalline lipid to the surface would lower the free energy of the system (see Fig. 7). Further addition of liposomal material can occur at every sticky collision. If a wide strip of liposomal bilayer were added, the folds at the edges of

Fig. 5. Freeze fracture electron micrograph of a sample quenched from room temperature in an aqueous solution of 10% glycerol. Note the large aqueous spaces between successive wraps of the tubular bilayers. Scale bar, 1 μm.

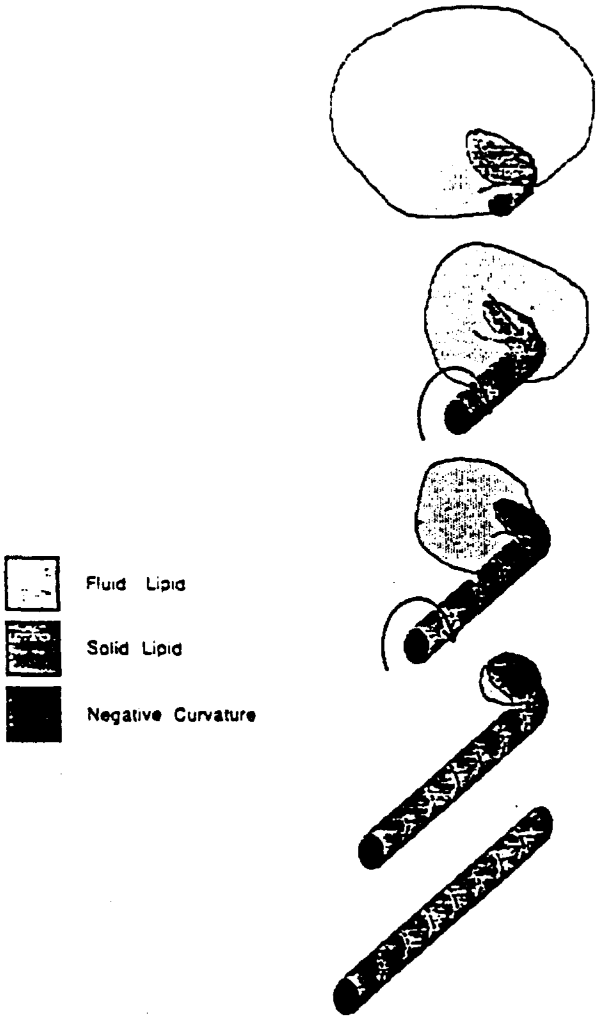

Fig. 7. Schematic representation growth of a tubule from a liposome resulting in a left-handed spiral. The tubule is rotating relative to the liposome. For simplicity the object which caused nucleation of the process has been omitted.

the bilayers could be far apart, and create both left- and right-handed spirals as in Fig. 8.

While it is clear that tubules can grow from liposomes without diffusion of monomer through solution, it is quite possible to form them in such a manner. $DC_{23}PC$ is precipitated from organic solution by addition of a miscible non-solvent, allowing tubule formation without liposomal intermediates [6]. Both methods produce nearly identical structures, with the exception that precipitation can also produce helices of similar dimensions. The helices are exclusively right-handed, whereas those formed from D-$DC_{23}PC$ are left-handed and those from the racemic lipid are both right- and left-handed [25]. The walls of the tubules formed by precipitation are frequently no more than one bilayer in thickness. The fact that there are two distinct formation routes for tubules argues that the tubular structure is thermodynamically stable, and is not a metastable morphology that forms because of the requirement that the lipids originate in large liposomes. Furthermore, any changes in area that may occur on chain crystallization that facilitate tubule formation are incidental to the ultimate stability of the microstructure.

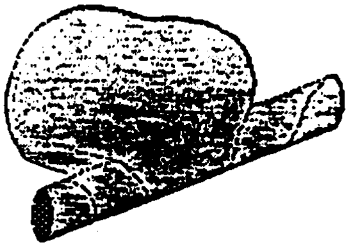

Fig. 8. Schematic wrapping of a liposome around a tubule. As the remaining flattened liposomal material becomes less wide than the full length of the tubule, two folded bilayer edges are left on the surface, producing both a left- and right-handed spiral as well as large sheets of tubular material between.

In summary, we have confirmed the connectivity of liposomal and tubular bilayers during the process of conversion from liposomes to tubules. This evidence of growth by wrapping, combined with evidence for unusually thin crystalline bilayers, suggests that growth of tubules from liposomes may in part be a consequence of a uniaxial increase in the area of the bilayer on cooling from the melt. This crystal structure may also favor a helicial curvature of the bilayer leading to the regular tubular internal diameter of about 0.75 μm.

The lipid described here is not unique in forming tubular structures. We have previously discussed [2] the great similarity between $DC_{23}PC$ tubules and structures formed from a positively charged non-polymerizable glutamate-based lipid [26]. We have also found that many diacetylenic phosphatidylcholines other than $DC_{23}PC$ form tubules [5,27]. It remains to be determined what these two different classes of surfactants have in common that stabilizes the tubular and helical structures.

As there are few convenient ways to manufacture objects with precise dimensions in the submicron size range, the ability of lipids to self-organize into a regular structure such as this is sure to be exploited. The polymerizability of diacetylenic lipids, which renders tubules structurally more stable even after dehydration, makes them even more attractive for applications that will require them to be processed and transferred into different media. Tubules can also be coated with a variety of metals (Calvert et al., unpublished), which renders the structure far more stable and useful for such applications as structural composites. We are continuing our pursuit of a better understanding of tubule formation, and the hypotheses presented will be tested by comparing the structure of bilayers of normal and tubule-forming lipids.

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